CHARACTERIZATION OF TWO FLAGELLA-RELATED PROTEINS FROM *CAULOBACTER CRESCENTUS*

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1. Introduction

The cell cycle of a stalked bacterium, *Caulobacter crescentus*, is divided into pre-synthetic, synthetic and post-synthetic periods, depending on the capacity of deoxyribonucleic acid (DNA) synthesis [1,2]. A single flagellum of this organism is formed at the post-synthetic period when DNA synthesis is completed and the cell enters into the predivisional state. This flagellum is also localized at one polar end of a swarmer cell and released into culture medium at the pre-synthetic period when the swarmer cell undergoes transition, without DNA synthesis, into a stalked cell. The flagella formation in the cell cycle is thus under temporal control and site-specific, and currently viewed as a good landmark for the study of specific gene expression and localization of morphogenesis in differentiating cells [3].

Two major proteins were found in different amounts in flagella released into culture medium [4], and the synthesis of these proteins occurred at the period of flagella formation [5]. These observations suggest either that the *Caulobacter* flagella filaments consist of two subunit proteins or that flagella filaments of different subunit proteins are formed simultaneously in the cell cycle. In the past studies, however, the putative flagellins have not been isolated separately in native forms and uncertainty still remained for the nature of these proteins.

We describe here the separate isolation of the two major proteins from released flagella of *C. crescentus* CB13. These two proteins could form filamentous polymers individually and elongate short core polymers of the heterogeneous proteins into long filaments. The amino acid compositions of these flagella proteins were quite similar.

2. Materials and methods

2.1. *Bacterial strain and growth conditions*

Caulobacter crescentus CB13bla wild type was used. The strain was grown reciprocally at 30°C in polypeptone–yeast extract (PYE) nutrient broth [6].

2.2. *Purification of flagellins*

C. crescentus CB13 was grown in PYE medium to the late stationary phase until the proportion of motile cells decreased to $< 5\%$ of the whole cell population. The culture was centrifuged at 12 000 rev./min for 10 min. To 6 l supernatant were added 30 ml 1.0 M phosphate buffer (pH 7.0), 7.5 ml 0.4 M ethylenediaminetetraacetate, (pH 7.0), and 857 g $(NH_4)_2SO_4$. The mixture was kept at 0°C overnight and centrifuged at 12 000 rev./min for 40 min at 2°C. The sediment was suspended in 70 ml 0.05 M tris(hydroxymethyl)aminomethane (Tris)–HCl, (pH 8.1) and reprecipitated similarly with 1.0 M $(NH_4)_2SO_4$. The precipitates were then resuspended in 4.4 ml 0.02 M Tris–HCl, (pH 8.1), dialyzed overnight against the same buffer at 4°C, sonicated briefly for 5 s (at setting 50 on Artek Sonic Dismembrator, NY) and centrifuged at 5000 rev./min for 10 min. The supernatant was crude flagella preparation. The crude flagella solution was adjusted to pH 2–3 with 2 N HCl, kept at room temperature for 1 h, and centrifuged at 6000 rev./min for 10 min and then at 30 000 rev./min for 1 h at 10°C in a Hitachi RP50

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rotor. The HCl extract was dialyzed against 0.02 M Tris–HCl (pH 8.1) at 4°C, charged onto 1.0 × 2.5 cm column of DEAE-cellulose (Wako Chemicals Co., Tokyo) equilibrated with 0.02 M Tris–HCl (pH 8.1) and eluted stepwise with 0.025 M NaCl (flagellin A) and 0.05 M NaCl (flagellin B) in the same buffer. After precipitation with 2.8 M $(NH_4)_2SO_4$, flagellins A and B were suspended in a small volume of 0.02 M Tris–HCl (pH 8.1) and dialyzed against the same buffer. The yields of flagellins A and B were 4.2 mg and 0.8 mg, respectively from 6 l culture.

2.3. *Polymerization of flagellins*

Before polymerization reaction, flagellin samples prepared as above were dialyzed against 0.01 N HCl and then against 0.02 M Tris–HCl (pH 8.1).

2.3.1. Short core polymers

Flagellin, 200 μl, 0.65 mg/ml, 5 μl 2 M NaCl and 50 μl 4 M $(NH_4)_2SO_4$ were incubated at 30°C for 15 h and centrifuged at 30 000 rev./min for 1 h in a Hitachi RP50 rotor. The sediment of short core polymers was washed twice with 0.2 ml 0.02 M Tris–HCl (pH 8.1) and suspended in 50 μl of the same buffer.

2.3.2. Long polymers

Flagellin, 100 μl, 0.65 mg/ml, 5 μl 1 M NaCl and 1.4 mg solid Na_2SO_4 were incubated at 30°C for 15 h.

2.3.3. Elongation of short core polymers

The above short core polymers, 10 μl, 90 μl flagellin 0.65 mg/ml, 5 μl 1 M NaCl and 7.5 μl 1.5 M Na_2SO_4 were incubated at 30°C for 15 h.

2.4. *Miscellaneous*

For the preparation of short fragments of native flagella, the above crude flagella were sonicated for 25 s at setting 50 on Artek Sonic Dismembrator, NY. The basic procedures for electron microscopy were as in [7]. Sodium dodecyl sulfate (SDS)–10% polyacrylamide slab gel electrophoresis was carried out as in [8]. The amino acid analysis was performed on a Durrum Analyzer model D-500. Fraction of flagellins was dialyzed extensively against 0.02 M sodium phosphate (pH 7.0) dried up in vacuo and subjected to 24 h and 72 h hydrolysis in 6 N HCl at 110°C. The value obtained for serine and threonine was determined by extrapolation to 0 h. Half-cystine was determined on a sample which was performic acid-oxidized prior to acid hydrolysis.

3. Results

3.1. *Separation of flagellins*

The HCl extract from crude flagella preparation contained two major proteins (fig.1). The molecular weight as estimated with various protein markers in SDS–10% polyacrylamide slab gel electrophoresis was 26 000 for the smaller protein (flagellin A) and

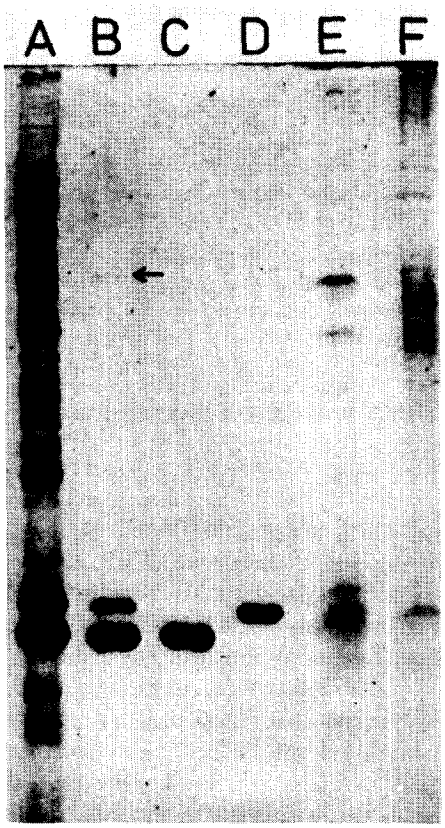


Fig.1. SDS–polyacrylamide slab gel electrophoresis of flagellins at various stages of purification. The purification procedures are in section 2. (A) Crude flagella; (B) HCl extract; (C,D) flagellins A and B, respectively, from DEAE-cellulose as described in fig.2 legend; (E) 1.0 M NaCl eluate; (F) 0.01 N HCl eluate. The arrow indicates the minor protein in the HCl extract.

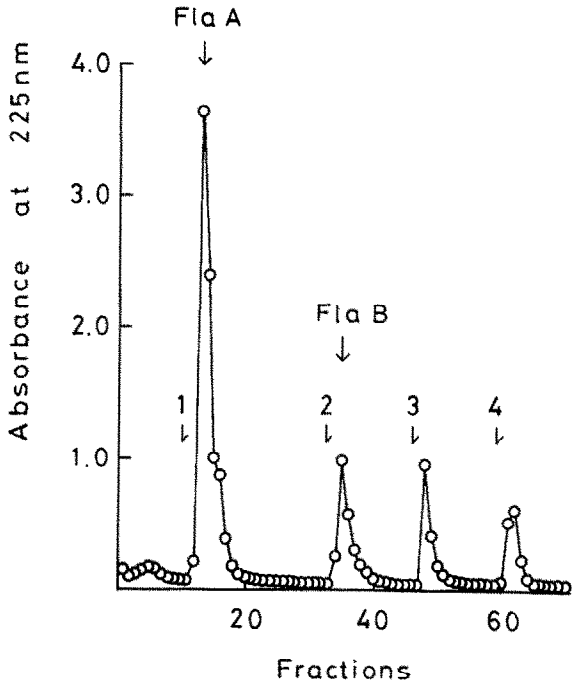


Fig.2. DEAE-cellulose column chromatography of flagellins. The procedures are in section 2. The HCl extract (6.1 mg protein) from crude flagella was used. The elution was stepwise with 0.025 M, 0.05 M and 1.0 M NaCl in 0.02 M Tris–HCl (pH 8.1), and 0.01 N HCl at the positions 1, 2, 3 and 4, respectively. The end absorbance at 225 nm was measured. The arrows indicate the elution positions of flagellins A and B.

28 500 for the larger protein (flagellin B). Flagellins A and B were eluted stepwise with 0.025 M and 0.05 M NaCl, respectively, from DEAE-cellulose (fig.2). The protein recovery was ~82%. Flagellin A was larger in quantity and the ratio of flagellins A and B ranged from 3–4 in different preparations. A minor protein was always present with these flagellins in the HCl extract and eluted with 1 M NaCl from DEAE-cellulose (fig.1,2). It is likely that this minor protein is derived from the hook structure that is attached to released flagella.

Table 1
The amino acid compositions of the *C. crescentus* CB13 flagellins A and B

	Flagellin A (mol/26 000 g)	Flagellin B (mol/28 500 g)
Aspartic acid, asparagine	34.0	36.0
Threonine	31.0	35.8
Serine	24.9	25.4
Glutamic acid, glutamine	19.8	24.9
Proline	0	0.8
Glycine	19.2	19.9
Alanine	37.7	38.9
Half-cystine	0	0
Valine	8.6	10.5
Methionine	1.1	1.5
Isoleucine	12.7	10.4
Leucine	31.1	36.7
Tyrosine	0	0
Phenylalanine	8.0	8.9
Tryptophan	n.d.	n.d.
Lysine	17.8	18.9
Histidine	2.3	2.4
Arginine	4.0	3.4

n.d., not determined

Numbers of residues per mole of protein monomer were calculated assuming mol. wt 26 000 for flagellin A and mol. wt 28 500 for flagellin B

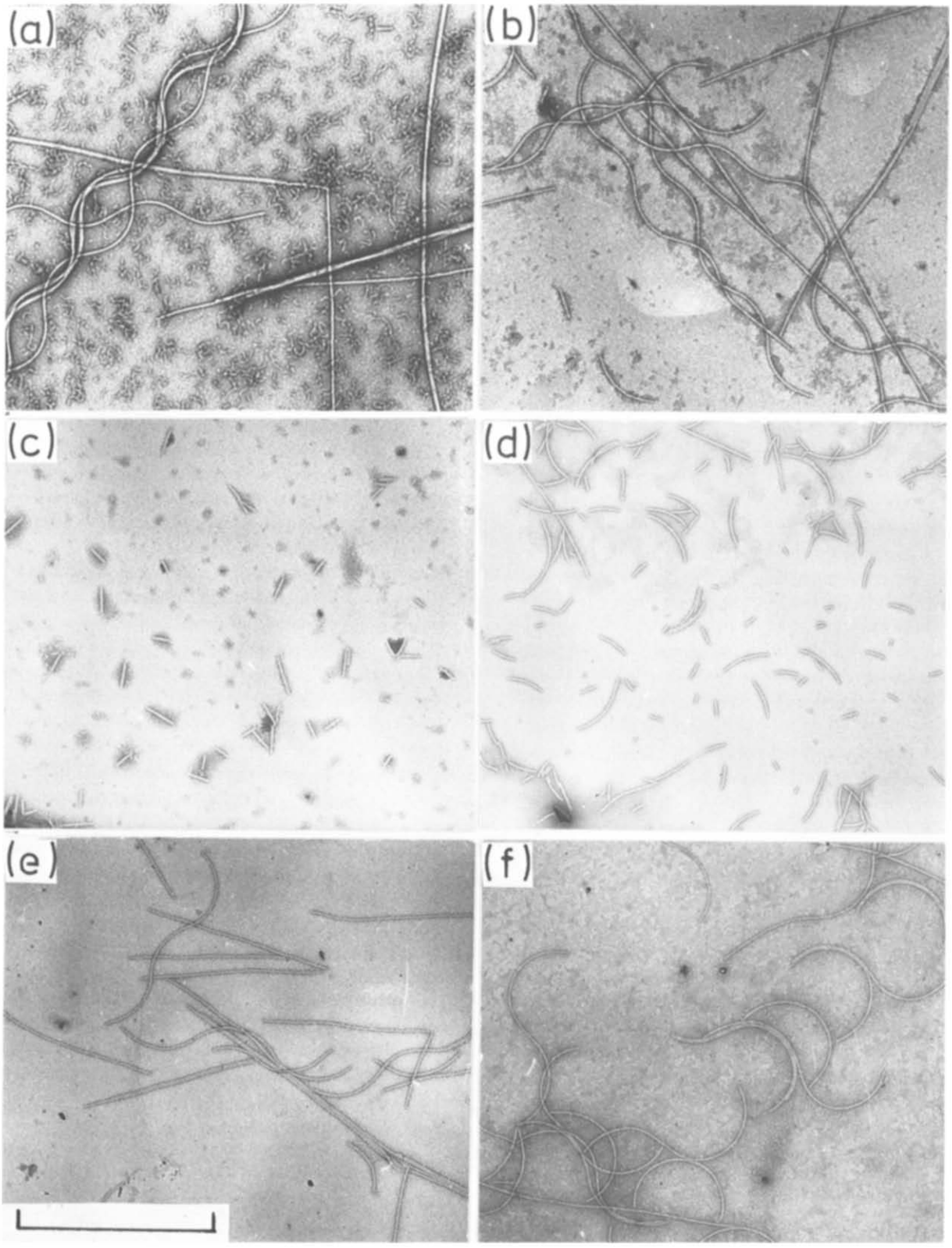
3.2. *Amino acid compositions*

The amino acid compositions of flagellins A and B were quite similar (table 1). The predominant residues were alanine, aspartic acid (including asparagine), leucine and threonine. Cysteine and tyrosine were not present in both proteins, and no amino acid residue except isoleucine in flagellin A was larger in number than in flagellin B. In several determinations, isoleucine residue was slightly larger in number in flagellin A. The number of arginine residues appeared to be the same in both proteins.

3.3. *In vitro polymerization of flagellins*

The isolation of the above two proteins (flagellins A and B) from released flagella indicates that these

Fig.3. Electron micrographs of flagellin polymers: (a) and (b), flagellins A and B polymers, respectively, upon addition of solid Na_2SO_4 to 0.1 M; (c) and (d), short core polymers of flagellins A and B, respectively; (e), filaments formed upon elongation of flagellin A core polymers with flagellin B monomers; (f), filaments formed upon elongation of flagellin B core polymers with flagellin A monomers. The bar represents 1.0 μm.


(a)
(b)
(c)
(d)
(e)
(f)

proteins are involved in the formation of flagella filaments. It was not clear from [4,5], however, whether these proteins form filamentous polymers jointly or independently, or one of the proteins is somehow required for the polymerization of the other protein.

Both flagellins A and B formed short polymers at high ionic strength, in 0.8 M $(NH_4)_2SO_4$ or Na_2SO_4 (fig.3c,d). The addition of solid Na_2SO_4 to the final concentration was required for the formation of long filaments directly from the flagellin monomers and under the conditions used, an appreciable proportion of these filaments were straight. Similar filamentous polymers were formed when the mixture of both flagellins A and B was used under the same conditions (data not shown).

Since flagellins A and B were capable of independently forming filamentous polymers, it became of interest to examine the elongation of short flagellin polymers by different flagellins. Short core polymers were prepared as described above and mixed with flagellin monomers at a protein ratio of core polymers 1 to monomers 4 in 0.1 M Na_2SO_4. Flagellin core polymers were elongated to long filaments by flagellin B monomers while flagellin B core polymers were elongated similarly by flagellin A monomers (fig.3e,f). The elongated polymers were of the mixture of wavy and straight filaments. As expected, flagellin A and B core polymers were elongated similarly by respective flagellin monomers (data not shown). It should be noted here that the elongation of flagellin B core polymers by flagellin A or B monomers consistently gave rise to more of wavy filaments than that of flagellin A core polymers under the conditions used. When short fragments of native flagella were used as the cores for the polymerization of flagellins A and B, the resultant long polymers were mostly wavy flagella filaments (data not shown). It thus appears that the morphology of the filaments formed depends on the core polymers used.

The above results show that the two proteins, flagellins A and B, from released flagella can be polymerized independently and jointly into filamentous polymers and indicates that both proteins are involved in the filament formation of native flagella as the subunits.

4. Discussion

We have described here the isolation of two flagellins from the *C. crescentus* CB13 flagella released in the culture medium, and their in vitro polymerization into flagella filaments. These flagellins A (mol. wt 26 000) and B (mol. wt 28 500) existed in rather constant but different quantities. The ratio of these flagellins did not change appreciably through the purification processes and the presence of a serine protease inhibitor, phenylmethylsulfonylfluoride, did not affect the outcome (unpublished data). Thus it is unlikely that the two flagellins arise through partial proteolysis of the larger flagellin during the purification processes.

The results of the in vitro polymerization indicate:

(i) Flagellins A and B can independently form long filamentous polymers;
(ii) Each flagellin does not require the other flagellin for polymerization;
(iii) The short polymers of each flagellin can be elongated into long filaments by the other flagellin.

The in vitro polymerization into long filaments is a conclusive evidence for the nature of these flagella proteins, particularly for flagellin B for which uncertainty still remained in the past studies. At the moment, however, the mode of in vivo polymerization into native flagella filaments remains for further study.

The amino acid compositions of flagellins A and B were quite similar (table 1). Cysteine and tyrosine were not present in either flagellin. The absence of proline and the presence of histidine in flagellin A are not consistent with [4,9]. The amino acid compositions reported in the previous papers were widely different. The amino acid compositions of flagellins A and B purified as described above were reproducible in several determinations. No amino acid residue except isoleucine in the smaller flagellin A was larger in number than in the larger flagellin B. The quite similar amino acid compositions might reflect a common evolutionary origin of these flagellins.

The occurrence of two flagellins poses an interesting problem of their expression in the *Caulobacter* cell cycle. Two distinct flagellin genes might be expressed differentially in the same cell, a structural modification is involved after transcription or translation. The latter possibility should still be considered despite the isoleucine residue number which was larger in the smaller flagellin A (table 1). Alternatively,

the cell is in two different phases of flagellin synthesis at a given ratio in the *Caulobacter* cell cycle and flagellins A and B are not synthesized in the same cell.

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